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Chromosomal Location of the Major Ribosomal RNA Genes in *Crassostrea virginica* and *Crassostrea gigas*

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Abstract. The chromosomal location of the major ribosomal RNA genes (rDNA) was studied in the eastern oyster *Crassostrea virginica* (Gmelin, 1791), and the Pacific oyster *C. gigas* (Thunberg, 1793) by fluorescence in situ hybridization (FISH). Probes were made by PCR amplification of the intergenic transcribed spacers between the 18S and 5.8S (ITS1), and between 5.8S and 28S RNA genes (ITS2), and labeled by PCR incorporation of DIG-11-dUTP. FISH with either probe on interphase nuclei showed two strong signals in both species. In *C. virginica*, FISH signals were located on the short arms of Chromosome 2, the second longest, with a centromere index of 0.39. In *C. gigas,* the rDNA probe hybridized to the long arms of Chromosome 10, the shortest chromosome. In both species, the FISH signals were confined to the telomere region of the chromosomes. All *Crassostrea* species have a haploid number of 10, and *C. virginica* and *C. gigas* share an almost indistinguishable karyotype. The size of the rDNA-bearing chromosome is the first major difference reported between the two karyotypes. This result suggests that considerable chromosomal evolution is possible in oysters despite the highly conserved haploid number.

INTRODUCTION

Oysters are important species for aquaculture, supporting major industries worldwide. The need for genetic analysis and improvement in oysters is great. At present, we know little about the genome of oysters including its most basic units—chromosomes. Chromosome characterization and identification remain difficult in oysters and most other marine mollusks. Chromosome identification is necessary for studies on aneuploids, chromosomal assignment of genes, and the development of chromosome-specific libraries and markers. Trisomic families have been produced in the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793) (Guo et al., 1998); and their identification and analysis have been difficult.

Oysters appear to have highly conserved karyotypes. All species of *Crassostrea* studied so far have a haploid number of 10 chromosomes (Nakamura, 1985). Although the haploid number is low, oyster chromosomes are small and similar in size and arm ratio, and do not permit traditional banding analyses. One banding study has been reported in the eastern oyster *C. virginica* (Gmelin, 1791) with limited resolution (Rodriguez-Romero et al., 1979). C- and G-banding patterns have been produced in *C. gigas*, but the reproducibility is low (Guo et al., unpublished). Recently, fluorescence in situ hybridization (FISH) has been used to characterize oyster chromosomes. A satellite DNA sequence has been assigned to two chromosomes of *C. gigas* (Clabby et al., 1996). The vertebrate telomere sequence, $(TTAGGG)_n$, has been localized to telomeres of *C. gigas* (Guo & Allen, 1997).

As part of our effort to characterize oyster chromosomes, we studied the location of major ribosomal RNA genes (or rDNA) in *C. virginica* and *C. gigas* with FISH. The major rDNAs in animals are present in tandem repeats of hundreds to thousands of units, and each unit contains the 18S, 5.8S, and 28S genes and intergenic spacers (Lewin, 1994). Ribosomal DNA has been assigned to specific chromosomes with FISH in many plants and animal species (De La Rúa et al., 1996). In some oysters, the location of rDNA has been studied indirectly by silver-staining of the nucleolar organizer re-

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gions (NORs), and intraspecific variation in the number and size of NORs has been observed (Thiriot-Quiévreux & Insua, 1992; Li & Havenhand, 1997). Intraspecific variation in NORs is common in fish and marine bivalves (Gold, 1984; Thiriot-Quiévreux & Insua, 1992). This variation may be caused by artifacts or differences in gene expression and/or DNA sequence, and FISH analysis with rDNA probes should be helpful. Here we report, for the first time, the localization by FISH of major rDNA on chromosomes of two species of *Crassostrea*.

MATERIALS AND METHODS

The *C. virginica* used for this study were from the Rutgers NEH strain. This strain has been selectively bred for over 36 years, primarily for disease-resistance. The *C. giga* used were from a stock that was originally introduced from Washington State and has been maintained in a quarantine system at Rutgers for five generations. Metaphase chromosomes were obtained from early embryos as described by Guo et al. (1992). Briefly, eggs and sperm were collected from mature oysters by stripping gonads. Eggs were passed through a 70-μm nytex screen to remove large tissue debris, and rinsed on a 20-mm screen. Eggs were resuspended in seawater and fertilized by adding sperm suspension. Excessive sperm were removed at 15 min post-fertilization by rinsing fertilized eggs on a 20-μm screen. Embryos were resuspended and cultured at 25°C. After 5 to 6 hours of culture, embryos were harvested into a 15 ml tube and treated with 0.005% of colchicine for 30 min. Colchicine was removed by pelleting out embryos with centrifugation. Nine parts of 0.075 M KCl were added to every part of concentrated embryos, and the hypotonic treatment lasted for 10 min. Embryos were then fixed in 1:3 acetic acid and methanol and stored at 4°C. For slide preparation, two to three drops of embryo suspension were loaded onto a slide and air-dried. Slides were stored at −20°C until FISH.

Intergenic transcribed spacers between the 18S and 5.8S (ITS1), and between 5.8S and 28S RNA genes (ITS2) were amplified and labeled by PCR, and used as FISH probes. PCR primer sequences are 5′-GGTTTCTGTAGGTGAACCTGC and 5′-CTGCGTTCTTCATCGACCC for ITS1, and 5′-GGGTCGATGAAGAACGCAG and 5′-GCTCTTCCCGCTTCACTCG for ITS2. Probes were labeled with digoxigenin-11-dUTP (alkali-stable) by PCR incorporation. Digoxigenin-11-dUTP and all other PCR reagents were purchased from Boehringer Mannhem (Indianapolis, Indiana). PCR reactions were performed in 25 μl volume containing 1 × PCR buffer with 1.5 mM of $MgCl_2$, 0.4 mg/ml of BSA, 0.2 mM each of dATP, dCTP, and dGTP, 0.13 mM of dTTP, 0.07 mM of Digoxigenin-11-dUTP, 0.5 U of Taq DNA polymerase, 0.2 μM of each primer, and 1 μg of oyster genomic DNA. Thermal cycling parameters were 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1 min at 72°C. The PCR product was purified through a G-50 column to remove unincorporated nucleotides.

All FISH reagents were purchased from Oncor, Incorporated. (Gaithersburg, Maryland). FISH was conducted according to the protocol recommended by Oncor and as described by Guo & Allen (1997). To prepare the probe for hybridization, 1.5 μL of the purified PCR product was added to 30 μL of Hybrisol VI (Oncor) and incubated at 72°C for 5 min. Slides carrying metaphase spreads were incubated in 2 × SSC at 37°C for 30 min, dehydrated for 2 min each in 70%, 80%, and 95% EtOH, and air-dried. Slides were then denatured in 2 × SSC containing 70% deionized formamide at 72°C for 2 min, dehydrated for 2 min each in cold 70%, 80%, and 95% EtOH, and air-dried. The denatured probe (30 μL) was added to the slides and covered with 22 × 50 mm glass coverslips. After sealing with rubber cement, the slides were incubated at 37°C in a humidified chamber overnight. Coverslips were then removed, and slides were washed in 1× SSC at 72°C for 5 min and 1 × PBD at room temperature for 2 min. Fluorescein-labeled anti-digoxigenin antibody (60 μL) was added to the slides, covered with plastic coverslips, and incubated at 37°C for 10 min in a humidified chamber. Slides were then washed three times for 2 min each in 1 × PBD at room temperature in the dark. Propidium iodide/antifade (10 μL) was added to each slide and covered with glass coverslips. Hybridization signals were analyzed and documented using a 3CCD camera and the Image-Pro Plus image system. Centromeric index was calculated as the length of short arm divided by total length (Levan et al., 1964).

RESULTS

PCR amplification in *C. virginica* generated a single product with both primer sets, ITS-1 and ITS-2. In the absence of digoxigenin-11-dUTP, ITS-1 generated an approximately 500 bp product, and ITS-2 generated a 750 bp product (Figure 1, lanes 2 and 3). The incorporation of digoxigenin-11-dUTP significantly decreased the mobility of both products, with the ITS-1 product shifted to 650 bp and the ITS-2 product to 900 bp (Figure 1, lanes 4 and 5). Production of ITS-1 probe was more reliable probably because of the smaller fragment size than ITS-2. Cleaning of labeled probes with columns clearly reduced background signals from FISH.

The embryo material was adequate for FISH analysis. Early embryos with four to eight cells contained a large amount of yolk material, which gave high background signals. Most of the embryos used in this study were between 30 and 120 cells and actively dividing. Four to 10 good metaphases were often obtained per slide.

In situ hybridization with ITS-1 probe to interphase nucleus consistently produced two clusters of signals per nucleus in *C. virginica* (Figure 2A). FISH signals within a cluster appeared to be discontinuous or fragmented.

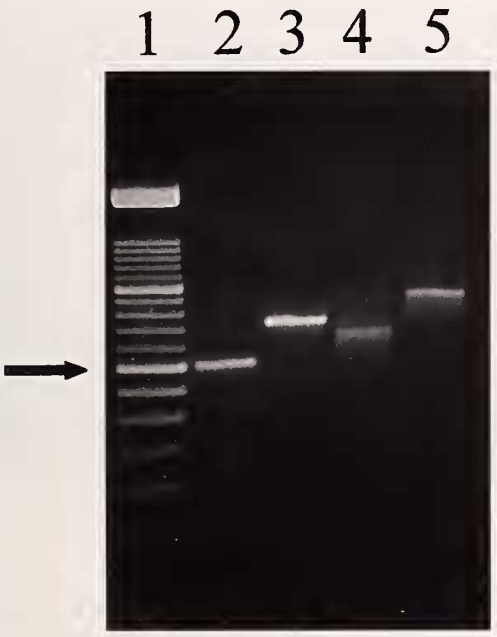

Figure 1. Products of PCR amplification of intergenic transcribed spacers between 18S and 5S (ITS1), and 5.8S and 28S RNA genes (ITS2), and the labeled fragments in *Crassostrea virginica:* Lane 1, 100 bp ladder; Lane 2, ITS1 product; Lane 3, ITS2 product; Lane 4, labeled ITS1; Lane 5, labeled ITS2.

FISH of ITS-1 on metaphase chromosomes showed that the signals were located on the telomeric region of the short arms of one pair of homologous chromosomes (Figure 2B). To characterize the hybridized pair of chromosomes, homologous chromosomes from six metaphases were paired, measured, and numbered as Chromosome 1 to 10 according to their length (Table 1). The FISH signals were clearly located on Chromosome 2 (Figure 2C,E), which was the second longest and had a centromeric index of 0.39. Chromosomes 1, 2, and 10 were clearly distinguishable from others because of their length and arm-ratio. The pairing of the other homologous chromosomes was less certain. FISH with the ITS-2 probe produced exactly the same pattern of signals that was indistinguishable as with ITS-1 (data not shown).

PCR amplification of ITS-1 in *C. gigas* produced a single fragment which is slightly longer than that from *C. virginica*. FISH with ITS-1 on *C. gigas* nuclei showed two clusters of signal per nucleus. Analysis of FISH signals on metaphase chromosomes revealed that signals are located in the telomeric region of the long arms of Chromosome 10, the smallest chromosomes (Figure 2D, E). FISH with ITS-2 produced the same results (data not shown). The FISH signal was often stronger in one of the homologous chromosomes, and the differential intensity was noticeable even when the two homologous chromosomes were closely situated in the metaphase (Figure 2D).

Table 1

Karyotype analysis of *Crassostrea virginica* chromosomes.

Chromosome number	Length as percent total length of haploid complement (± SD)	Centromeric Index[1] (± SD)
1	12.30 ± 0.23	0.48 ± 0.02
2	11.41 ± 0.09	0.39 ± 0.01
3	10.80 ± 0.09	0.42 ± 0.01
4	10.71 ± 0.12	0.48 ± 0.01
5	10.45 ± 0.11	0.41 ± 0.01
6	9.99 ± 0.19	0.41 ± 0.01
7	9.54 ± 0.22	0.38 ± 0.04
8	8.73 ± 0.24	0.48 ± 0.01
9	8.65 ± 0.14	0.36 ± 0.05
10	7.42 ± 0.14	0.48 ± 0.02

[1] Length of short arm divided by total length (Levan et al., 1964).

DISCUSSION

The major rDNA genes (18S, 5.8S, and 28S) are unambiguously assigned to chromosomes of *C. virginica* and *C. gigas*. This study provides the first chromosomal assignment of genes with FISH in oysters and possibly all marine mollusks. Protocols for chromosome preparation, probe labeling, and FISH used in this study are highly reproducible for oysters and should promote FISH analysis in other marine mollusks. Mollusks represent a major taxon for evolutionary studies, and information about molluscan genomes is important for comparative genomics.

Results of this study clearly show that there is only one site for the major rDNA cluster (18S, 5.8S, and 28S) in the two species, which confirms the previous observation of one pair of NOR-chromosomes in *C. gigas* (Thiriot-Quiévreux & Insua, 1992). No comparable data from *C. virginica* and other *Crassostrea* species are available. Two or more NOR-chromosomes have been observed in several *Ostrea* oyster species (Insua & Thiriot-Quiévreux, 1991, 1993; Thiriot-Quiévreux & Insua, 1992; Li & Havenhand, 1997) and three *Mytilus* mussel species (Insua et al., 1994).

Intraspecific variation in the number and size of NORs is common in fish and marine bivalves (Gold, 1984; Thiriot-Quiévreux & Insua, 1992). This variation may be caused by artifacts, differences in gene expression or DNA sequence, and has rarely been confirmed with FISH analysis. In *C. gigas*, no variation in the number of NOR-chromosomes has been observed, but the two homologous chromosomes differ in the size (or intensity) of NORs (Thiriot-Quiévreux & Insua, 1992). We also noticed that the FISH signals are often considerably stronger on one of the homologous chromosomes than the other

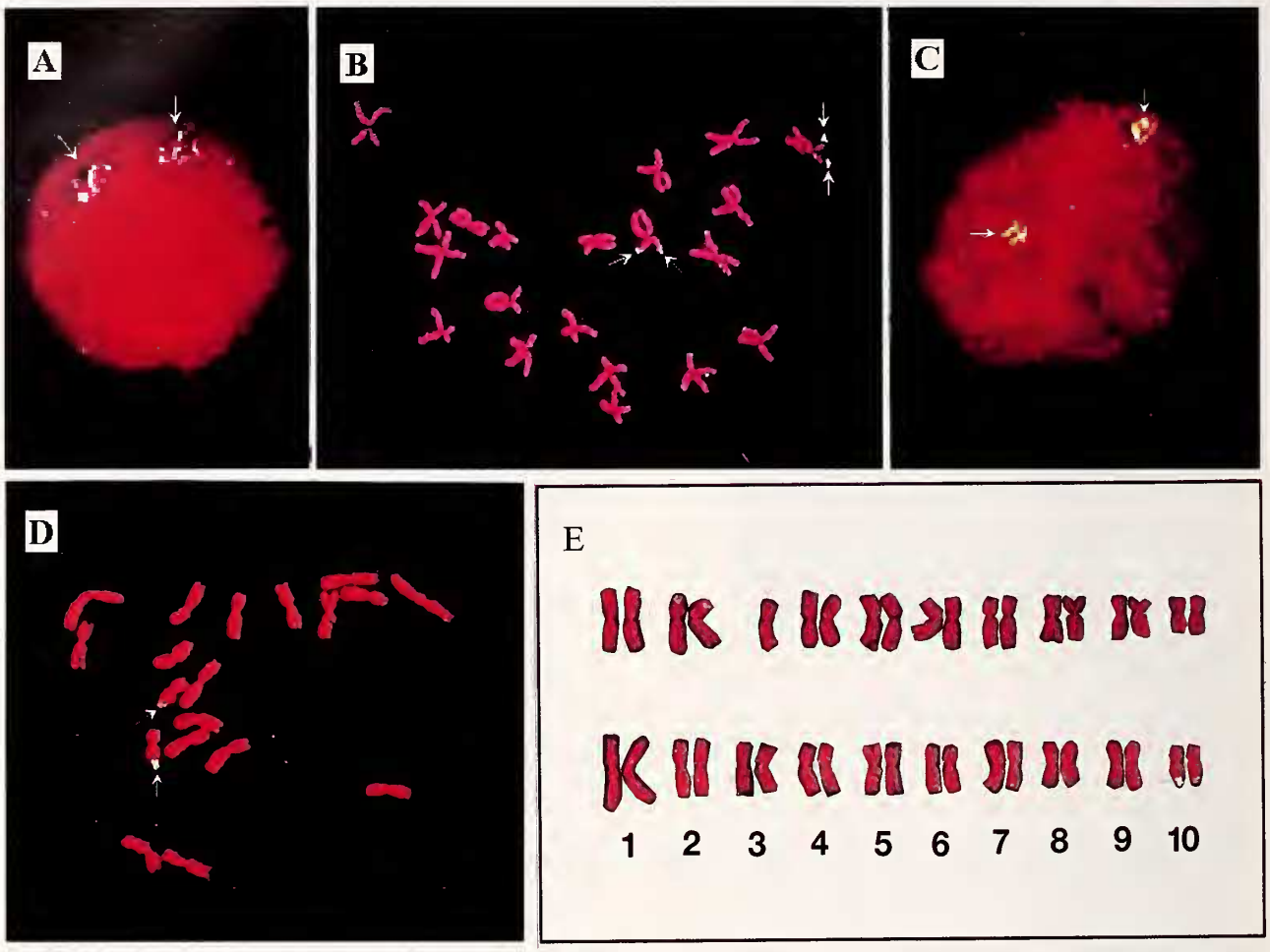

Figure 2. FISH with the rDNA probe (ITS1) on interphase nuclei and metaphase chromosomes in *Crassostrea virginica* and *C. gigas*: A. *C. virginica* nucleus; B. *C. virginica* metaphase; C. *C. gigas* nucleus; D. *C. gigas* metaphase; and E. karyotypes of *C. virginica* (upper) and *C. gigas* (lower), with rDNA probe hybridized to Chromosomes 2 and 10, respectively. Arrows indicate FISH signals.

(Figure 2D). The same is true with telomeric repeats in *C. gigas* (Guo & Allen, 1997). The differential intensity may be superficial and caused by random variation in FISH, or it may reflect true differences (loss or gain) in DNA sequence. Also, FISH signals in interphase nuclei were fragmented, which may suggest that there is significant space among the repetitive units or clusters of units. This phenomenon has also been observed in tiger beetles (De La Rúa et al., 1996).

Interspecific variation in rDNA or NOR location is expected and has been used for phylogenetic analysis (De La Rúa et al., 1996). Ribosomal DNA is found in telomeric regions of chromosomes in both oysters. In *C. virginica*, analysis of multiple karyotypes positively assigns the rDNA on the short arm of Chromosome 2, the second longest chromosome. Our karyotype for *C. virginica* closely resembles a previously described one in the same species, except that Chromosome 2 was named Chromosome 3 in the previous study, according to centromeric index, rather than total length (Longwell et al., 1967). In *C. gigas*, the rDNA is clearly located at the long arm of Chromosome 10, the smallest chromosomes, confirming results from NOR analysis (Thiriot-Quiévreux & Insua, 1992). The karyotypes of the two species are almost indistinguishable, and the difference in the rDNA-bearing chromosome is the first major difference between the two karyotypes recognized to date. This observation suggests that considerable chromosomal evolution is possible in oysters despite the highly conserved haploid number.

The rDNA FISH probe provides an effective method for the identification of Chromosomes 2 and 10 of *C. virginica* and *C. gigas*, respectively. We are in the process of developing additional chromosome-specific FISH probes for trisomic analysis in oysters (Guo et al., 1998).

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LITERATURE CITED

Clabby, C., U. Goswami, F. Flavin, N. P. Wilkins, J. A. Houghton & R. Powell. 1996. Cloning, characterization and chromosomal location of a satellite DNA from the Pacific oyster, *Crassostrea gigas*. Gene 168(2):205–209.

De La Rúa, P., J. Serrano, G. M. Hewitt & J. Galián. 1996. Physical mapping of rDNA genes in the ground beetle *Carabus* and related genera (Carabidae: Coleoptera). Journal of Zoological Systimatics and Evolutionary Research 34:95–101.

Gold, J. R. 1984. Silver-staining and heteromorphism of chromosomal nucleolus organizer regions in North American cyprinid fishes. Copeia 23:133–139.

Guo, X. & S. K. Allen, Jr. 1997. Fluorescence in situ hybridization of the vertebrate telomere sequence to chromosome ends of the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Shellfish Research 16(1):87–89.

Guo, X., K. Cooper, W. K. Hershberger & K. K. Chew. 1992. Genetic consequences of blocking polar body I with cytochalasin B in fertilized eggs of the Pacific oyster, *Crassostrea gigas*: I. Ploidy of resultant embryos. Biological Bulletin 183:381–386.

Guo, X., H. Yang, B. J. Landau, G. A. DeBrosse & S. K. Allen Jr. 1998. The creation of aneuploid families in the Pacific oyster, *Crassostrea gigas* Thunberg. Journal of Shellfish Research 17(1):328. [abstract only]

Insua, A. & C. Thiriot-Quiévreux. 1991. The characterization of *Ostrea denselamellosa* (Mollusca, Bivalvia) chromosomes: karyotype, constitutive heterochromatin and nucleolus organizer regions. Aquaculture 97:317–325.

Insua, A & C. Thiriot-Quiévreux. 1993. Karyotype and nucleolus organizer regions in *Ostrea puelchana* (Bivalvia: Ostreidae). The Veliger 36:215–219.

Insua, A., J. P. Labat & C. Thiriot-Quiévreux. 1994. Comparative analysis of karyotypes and nucleolar organizer regions in different populations of *Mytilus trossulus*, *Mytilus edulis* and *Mytilus galloprovinvialis*. Journal of Molluscan Studies 60:359–370.

Levan, A., D. Fredga & A. A. Sandberg. 1964. Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220.

Lewin, B. 1994. Gene V. Oxford University Press: Oxford, England

Li, X. X. & J. N. Havenhand. 1997. Karyotype, nucleolus organizer regions and constitutive heterochromatin in *Ostrea angasi* (Mollusca: Bivalvia): evidence of taxonomic relationships within the Ostreidae. Marine Biology 127:443–448.

Longwell, A. C., S. S. Stiles & D. G. Smith. 1967. Chromosome complement of the American oyster *Crassostrea virginica*, as seen in meiotic and cleaving eggs. Canadian Journal of Genetics and Cytology 9(4):845–858.

Nakamura, H. K. 1985. A review of molluscan cytogenetic information based on the CISMOCH-computerized index system for molluscan chromosomes. Bivalvia, Polyplacophora and Cephalopoda. Venus 44:193–226.

Rodriguez-Romero, F., A. Laguarda-Figueras, M. Uribe-Alcocer & M. L. Rojas-Lara. 1979. Distribution of "G" bands in the karyotype of *Crassostrea virginica*. Venus 38(3):180–184.

Thiriot-Quiévreux, C. & A. Insua. 1992. Nucleolar organizer region variation in the chromosomes of three oyster species. Journal of Experimental Marine Biology and Ecology 157: 33–40.